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14. ABSTRACT
The experiments carried out under this Innovator award allowed us to set up a genetic system to identify genes through loss of function (RNAi) or gain of function (over-expression) functional screens that are capable of influencing cells to acquire tumor-like properties. In this way we have identified many novel tumor suppressors and oncogenes. Many of these are found to be altered in breast cancer and other cancer types. Importantly, we developed the ability to identify genes whose inhibition is lethal in cancer cells but not in normal cells. We discovered that many genes cancer cells depend on are not oncogenes but genes never implicated or altered in cancers. This introduced the concept of non-oncogene addiction and opened many researchers to the idea that there are many different potential anti-tumor drug targets that are only going to be found through functional screens for cancer cell vulnerabilities. In addition we devised and tested a novel peptide display technology using synthetic biology to probe the auto-immune response of cancer patients to look for early diagnosis biomarkers. We have synthesized the human peptidome and developed methodology to determine what portions of human proteins are being recognized by auto-anibodies on a genomic scale.

15. SUBJECT TERMS
Oncogene addiction, non-oncogene addiction, REST, PVRL4, shRNA, RNAi, functional genomics, oncogenes, tumor suppression, beta-TRCP, synthetic lethality, genetic screening

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INTRODUCTION

The evolution of human cells into malignant derivatives is driven by the aberrant function of genes that positively and negatively regulate various aspects of the cancer phenotype, including altered responses to mitogenic and cytostatic signals, resistance to programmed cell death, immortalization, neoangiogenesis, and invasion and metastasis[(1)]. The integrity of these gene functions is compromised by substantial genetic and epigenetic alterations observed in most cancer cell genomes. To understand the tumorigenic process, it is imperative to identify and characterize the genes that provide tumor cells with the capabilities requisite for their initiation and progression. However, the identities of those genes that contribute to the tumor phenotype are often concealed by the frequent alterations in genes that play no role in tumorigenesis.

Identifying genes that restrain tumorigenesis (tumor suppressors) has proven especially challenging due to their recessive nature. Further complicating their discovery are the multifaceted mechanisms by which tumor suppressor genes are inactivated including changes in copy number and structure, point mutations, and epigenetic alterations[(2)]. Moreover, the mechanisms by which tumor suppressor genes are inhibited may vary between tumors. With this in mind, a variety of molecular and cytogenetic technologies have been used to establish extensive catalogs of genetic alterations within human cancers[(3,4)]. And while it is generally accepted that highly recurrent aberrations signify changes that are important for tumor development, the causal perturbations underlying tumor genesis are often confounded by the extensive size of alterations and the large number that are incidental to the tumor phenotypes. As such, new strategies to delineate genes with functional relevance to tumor initiation and development are essential to understanding these processes.

One approach to this problem involves the use of *in vitro* models of human cell transformation. In such models, primary cells are transformed into tumorigenic derivatives by the coexpression of cooperating oncogenes[(5-7)]. These experimental models have been useful in delineating the minimum genetic perturbations required for transformation of various human cell types as well as evaluating the functional cooperation between a gene of interest and a defined genetic context. To date, these models of human cell transformation have incorporated genes already implicated in human tumorigenesis. However, such models also provide a potentially useful platform for the identification of new pathways that contribute to the transformed phenotype.

In this award, we originally proposed two basic areas of investigation. The first area is the development of methods to investigate the repertoire of the immune system to determine whether auto-antibodies exist that might predict the onset of breast cancer. The second area was the construction and use of shRNA libraries to find genes relevant to breast cancer and hopefully targets that might kill tumor cells.

A key part of our research plan has been the development and use of retroviral vectors expressing RNA interference RNAs to identify human genes involved in causing or restraining cancer. In our first progress reports we described our efforts to develop shRNA libraries and showed they could be used to identify tumor suppressors. Ultimately our goal is to screen of complex pools of shRNA expressing retroviruses each marked with a bar code that allows the results of the screen to be read out by microarray hybridization. We demonstrated this could be accomplished in enrichment screens for shRNAs that caused cellular transformation and growth in soft agar and identified the REST gene, the INNP4B gene and several other tumor suppressors. We have expanded this analysis to a much deeper level.

In the one year extension we have continued some of the screening for genes whose knockdown causes transformation.

BODY

Identification of cancer-relevant genes.

RESULTS

PTPN12 suppresses transformation of human mammary epithelial cells

Signal transduction networks play a prominent role in the malignant behavior of cancer cells. To identify new networks that regulate cellular transformation in human breast cancer, we performed a genetic screen for kinases and phosphatases that suppress cellular transformation in genetically engineered human mammary epithelial cells (HMECs) (Figure 1A). HMECs isolated from healthy human breast tissue were transduced with lentiviruses expressing hTERT and SV40-Large T. These cells (herein termed TLM-HMECs) are immortal but do not proliferate in the absence of extracellular matrix. For the genetic screen, we generated a shRNA library targeting all human kinases and phosphatases (6 shRNAs per gene) in the context of our miR30-based retroviral vector. TLM-HMECs were transduced with the entire retroviral shRNA library (1000 proviral integrations / shRNA), and cultured in the absence of extracellular matrix for 3 weeks. shRNA-induced cellular transformation was indicated by the formation of anchorage-independent macroscopic colonies. We isolated 530 colonies from 2 independent screens, and identified proviral shRNAs by PCR-recovery and sequencing. shRNAs identified in both replicate screens were considered to target candidate suppressors of transformation. Several genes were targeted by multiple independent shRNAs including the well-documented tumor suppressors PTEN and.

The top-scoring candidate from this genetic screen was the protein tyrosine phosphatase PTPN12 (also known as PTP-PEST). Intriguingly, many tyrosine kinases have been shown to be important drivers of human cancer. In contrast, while in principle protein tyrosine phosphatases (PTPs) may antagonize proto-oncogenic TKs, the role of PTPs in human tumor suppression is poorly understood. Notably, PTPN12 has not been previously implicated in tumor suppression. Three independent shRNAs targeting PTPN12 exhibited robust cellular transformation in TLM-HMECs (Figure 1C). Importantly, the degree of depletion by each shRNA corresponded to the severity of the phenotype (Figure 1B and 1C). In addition, restoring PTPN12 expression with an exogenous PTPN12 cDNA completely suppressed transformation by PTPN12 shRNA (Figure 1D), thus ruling out RNAi off-target effects. Collectively, these data indicate that PTPN12 is a potent suppressor of transformation in mammary epithelial cells.

PTPN12 phosphatase activity is required for suppression of cellular transformation

To address the mechanism by which PTPN12 suppresses mammary cell transformation, we first determined whether the tyrosine-phosphatase activity of PTPN12 is required for this function. A point mutation was generated in the active site of PTPN12 (amino acid C231), which has previously been shown to ablate phosphatase activity. To determine whether this phosphatase-inactive mutant could suppress transformation in PTPN12 loss-of-function cells, an shRNA-resistant derivative of the PTPN12-C231S cDNA was transduced into TLM-HMECs expressing a PTPN12 shRNA. In contrast to wild-type PTPN12 (Figure 1D), the C231S mutant had no effect on HMEC transformation (Figure 1E), suggesting that the tyrosine-phosphatase activity of PTPN12 is required for suppressing transformation.

Figure 1

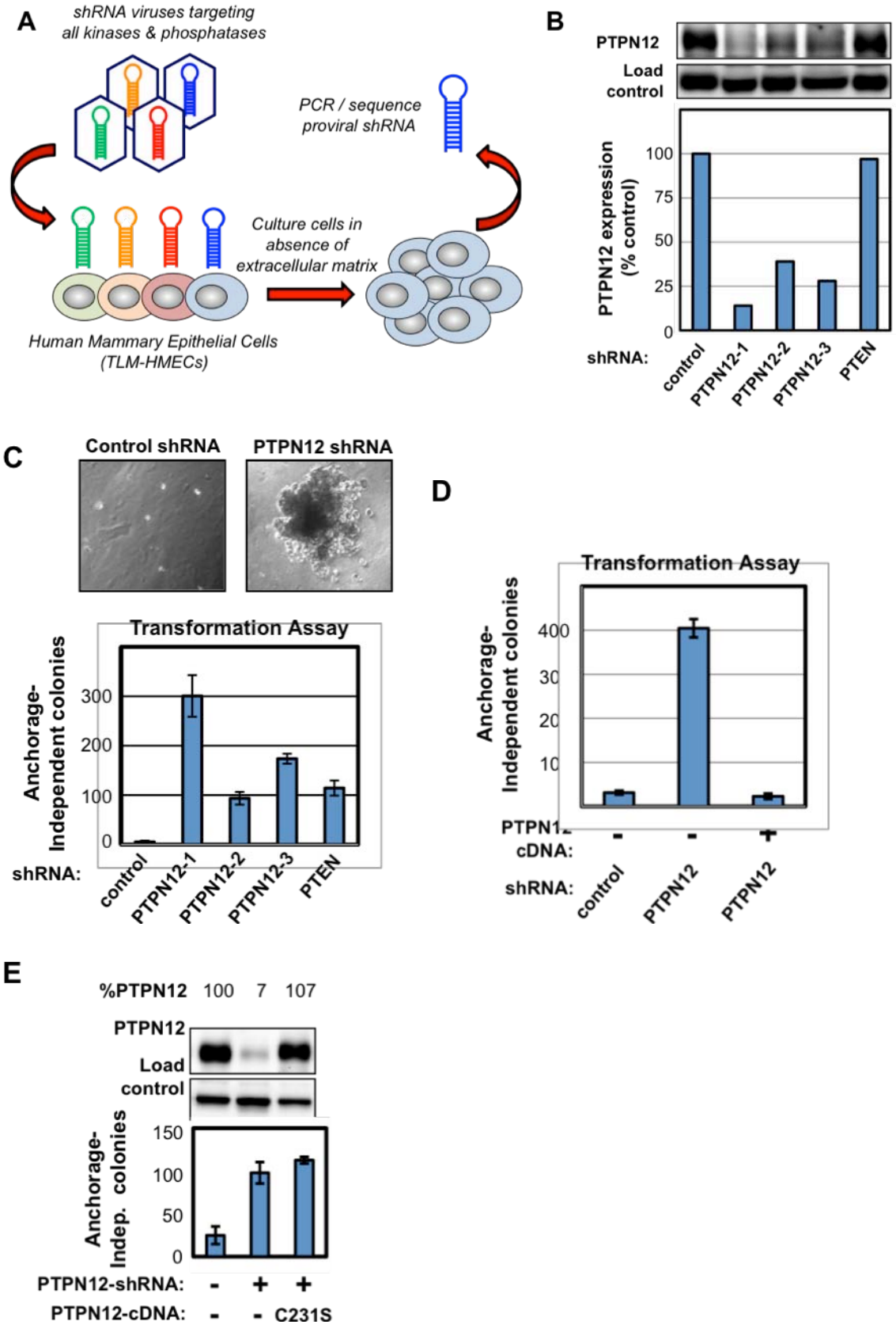
A
shRNA viruses targeting all kinases & phosphatases
PCR / sequence proviral shRNA
Culture cells in absence of extracellular matrix
Human Mammary Epithelial Cells (TLM-HMECs)
B
PTPN12
Load control
PTPN12 expression (% control)
100
75
50
25
0
shRNA:
control
PTPN12-1
PTPN12-2
PTPN12-3
PTEN
C
Control shRNA
PTPN12 shRNA
Transformation Assay
Anchorage-Independent colonies
300
200
100
0
shRNA:
control
PTPN12-1
PTPN12-2
PTPN12-3
PTEN
D
Transformation Assay
Anchorage-Independent colonies
400
30
20
10
cDNA:
shRNA:
control
PTPN12
PTPN12
E
%PTPN12 100 7 107
PTPN12
Load control
Anchorage-Indep. colonies
150
100
50
0
PTPN12-shRNA: - + +
PTPN12-cDNA: - - C231S

Figure 1. A Genetic Screen for Tumor Suppressors Identifies PTPN12.
(A) Schematic of genetic screen for suppressors of human mammary epithelial cell (HMEC) transformation. A pool of retroviral shRNAs targeting all human kinases and phosphatases (6 shRNAs/gene) was transduced into TLM-HMECs, and assessed for anchorage-independent proliferation. shRNAs were PCR-amplified and sequenced from macroscopic colonies. Two independent screens were performed.
(B) Depletion of PTPN12. PTPN12 protein expression in TLM-HMECs transduced with vectors expressing the indicated shRNAs. Normalized quantification of PTPN12 expression is shown in the lower panel.
(C) PTPN12 loss-of-function transforms TLM-HMECs. Anchorage-independent proliferation in TLM-HMECs transduced with control shRNA or shRNAs targeting PTPN12 or PTEN as indicated.
(D) Restoring PTPN12 expression suppresses transformation by PTPN12 shRNA. Anchorage-independent proliferation in TLM-HMECs transduced with control or PTPN12-shRNA in combination with PTPN12 cDNA as indicated.
(E) The enzymatic activity of PTPN12 is required to suppress cellular transformation. TLM-HMECs expressing a PTPN12 shRNA were transduced with lentivirus encoding control or shRNA-resistant PTPN12-C231S mutant cDNA and assessed for PTPN12 expression by western (top) and anchorage-independent proliferation (bottom).

Key research accomplishments

1. Discovery of the PTPN12 gene as a suppressor of cellular transformation.
2. Discovery that the tyrosine phosphatase activity of PTPN12 is needed for suppressing transformation and abnormal acinar growth.

Statement of Work.

This section has not changed from the previous final report.

Year 1

Task 1 (Months 1-12)
In the first year we anticipate beginning to work out the conditions for using the bar coding method to follow retroviruses containing hairpins as mixtures in complex libraries. We now have a library of 22,000 hairpins covering about 8,000 genes. We will be performing exploratory screens and optimizations to test the quality of the barcoding method. We must have this method working well to carryout the synthetic lethal screens.
We accomplished this goal in two ways. The first is we performed a bar code screen for potential tumor suppressors and identified several genes described in our first report and in Westbrook et al, 2005). Secondly, we have improved our vectors to allow single copy knockdown as described in Stegmeier et al. 2005. This was absolutely essential for the bar coding experiments we have proposed to kill cancer cells.

Task 2 (Months 1-24 and possibly longer, an ongoing effort)
We will continue to expand the library during this period to encompass more genes. This will be done in collaboration with Dr. Greg Hannon.
We have accomplished this goal by the generation of a second generation library in the mir30 context as described in Silva et al., 2005. This covers 140,00 human and mouse shRNAs as was described in last years report. We have also developed new and better knockdown vectors to allow us to knock down genes with greater penetrance. Right now

we feel we have nearly genome-wide coverage and are working on a new library which if successful will be a much better and more trustworthy library.

Task 3 (Months 6-24)
We also will begin the process of analyzing the human genome for coding sequences to set up the bio-informatics analysis to generate a list of sequences we wish to express to look for auto-antibodies. We should begin synthesizing oligo nucleotides to cover human genes.
We have designed oligonucleotides to cover the human genome. We are through cloning them in phage display vectors. We are characterizing the libraries and trying to figure out how best to screen them. We ran into the problem that screening them by microarray ran into cross hybridization problems which we are addressing bioinformatically.

Year 2.
Task 4 (Months 13-24)
In this period we plan to begin to carryout screen to look for genes which when knocked down by shRNA will interfere with the growth of cells containing defined mutations that lead to breast cancer. We will start with known tumor suppressors such as loss of p53 and Rb. We will use the barcoding methods. We may also screen for genes that sensitize cells to killing by gamma IR.
We initially tried PTEN mutants but were unable to find synthetic lethals. We have now successfully started with Kras and identified a few reproducible genes in a pilot experiment that are selectively toxic with Kras mutant cells.

Task 5 (Months 18-36)
We will begin to synthesize shRNA clones corresponding to the mouse genome.
We have completed this.

Task 6 (Months 12-24)
We will expand the library of short coding regions for the autoantibody project and work out conditions to express these protein fragments in bacteria in a high through-put fashion.
We have made the libraries and are working on developing methods to analyze the results.

Year 3.

Task 7 (Months 24-36)
We will continue to screen for synthetic lethals with tumor causing mutations relevant to breast cancer. In addition, by this time we will be retesting the synthetic lethal positives from the initial screens performed in year two.
We performed straight lethal and synthetic lethal experiments with ras. We have carried out one screen and now hope to examine out hits in breast lines with active and inactive ras.

Task 8 (Months 24-36)
We plan to work out the conditions for placing the proteins expressing short segments of human proteins for the auto-antibody screening project onto glass slides for screening purposes.
We have abandoned this aim in that we switched our approach to a phage display library which does not require glass slide. We made our first comprehensive library in a T7 display vector.

Task 9 (Months 24-36)
We will continue to characterize the mouse shRNA library.
We are characterizing the mouse library. It was transferred into our best knockdown vector. We are in the process of performing a screen in stem cells to look for genes that enhance ionizing radiation resistance or sensitivity.

Years 4 and 5.
These years are listed together as they will be consumed with executing the long-term goals of the Tasks outlined in years 1 through 3.

Task 10 (Months 36-60)
We will begin to screen human sera for autoantibodies against our arrays of human protein fragments. We will work out these methods and attempt to begin a higher through-put analysis to determine if common epitopes are eliciting a response in breast cancer patients.
We have made the libraries and obtained the sera samples. In this last year we have established that the methods should work with reconstruction experiments and are working out ways to screen the data generated. We cannot use microarray readouts because of cross hybridization. We have solved this by sequencing using Next Generation sequencing using the Solexa platform. We are in the process of performing the initial IPs to characterize the immunome's interaction with the human peptidome.

Task 11 (Months 36-60)
We will infect mice with retroviral libraries and screen for tumor suppressors in the breast and possibly other tissues.
We did not get to the point where we could do this aim as we are consumed with finding the cancer the lethals.

Task 12 (Months 36-60)
We will be examining the genes we have found in various screens using standard molecular biological approaches to understand their roles in control of the responses we screened for in previous tasks.
We are doing this with some of our tumor suppressor hits and some potential oncogenes we have found. We are following up on the cancer-specific lethals as well as ras synthetic lethals. The ras synthetic lethals appear to be falling into a pathway that reveals that ras is sensitive to mitotic perturbation.

REPORTABLE OUTCOMES

No new papers resulted from the extension. There was only $20 K left over for the no cost extension period.

CONCLUSIONS

Screens for Tumor suppressors using the RNAi library

We were able to work up a new potential tumor suppressor, PTPN12 which we think may antagonize endogenous oncogenic tyrosine kinases like Her2 and EGFR. It remains to be seen how frequently it is mutated in breast cancers.

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APPENDICES

None for this period.